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The effect of Me_2SO overexposure during cryopreservation on HOS TE85 and hMSC viability, growth and quality

Timothy J. Morris [a], Andrew Picken [a], Duncan M.C. Sharp [b], Nigel K.H. Slater [b], Christopher J. Hewitt [c], Karen Coopman [a, *]

[a] Centre for Biological Engineering, Department of Chemical Engineering, Loughborough University, Leicestershire, LE11 3TU, UK
[b] Department of Chemical Engineering and Biotechnology, University of Cambridge, Cambridge, CB2 3RA, UK
[c] Aston Medical Research Institue, School of Life and Health Sciences, Aston University, Aston Triangle, Birmingham, B4 7ET, UK

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ABSTRACT

With the cell therapy industry continuing to grow, the ability to preserve clinical grade cells, including mesenchymal stem cells (MSCs), whilst retaining cell viability and function remains critical for the generation of off-the-shelf therapies. Cryopreservation of MSCs, using slow freezing, is an established process at lab scale. However, the cytotoxicity of cryoprotectants, like Me_2SO, raises questions about the impact of prolonged cell exposure to cryoprotectant at temperatures >0 °C during processing of large cell batches for allogenic therapies prior to rapid cooling in a controlled rate freezer or in the clinic prior to administration. Here we show that exposure of human bone marrow derived MSCs to Me_2SO for ≥1 h before freezing, or after thawing, degrades membrane integrity, short-term cell attachment efficiency and alters cell immunophenotype. After 2 h's exposure to Me_2SO at 37 °C post-thaw, membrane integrity dropped to ~70% and only ~50% of cells retained the ability to adhere to tissue culture plastic. Furthermore, only 70% of the recovered MSCs retained an immunophenotype consistent with the ISCT minimal criteria after exposure. We also saw a similar loss of membrane integrity and attachment efficiency after exposing osteoblast (HOS TE85) cells to Me_2SO before, and after, cryopreservation.

Overall, these results show that freezing medium exposure is a critical determinant of product quality as process scale increases. Defining and reporting cell sensitivity to freezing medium exposure, both before and after cryopreservation, enables a fair judgement of how scalable a particular cryopreservation process can be, and consequently whether the therapy has commercial feasibility.

1. Introduction

Cell therapies hold the potential to revolutionise healthcare as regenerative medicines, replicating the success of the human therapeutic protein industry. However, cell therapies are more complex than protein therapeutics, which to the makes the preservation, long-term storage and shipment of cellular therapies a challenging prospect. Cryopreservation methods for long-term storage are described only in brief when reported in cell therapy clinical trial protocols. For example, while a cooling rate may be provided, processing times before freezing or after thawing are rarely given [13].

Components commonly used in lab-based cryopreservation protocols, including animal serum and dimethylsulfoxide introduce commercial, safety and regulatory risks for new therapies [38]. Animal-component free cryopreservation solutions are already available, such as the CryoStor® range from BioLife Solutions and PrimeXV®-FreezIS from Irvine Scientific. However, with cell-based therapeutics approaching the critical Phase III stage of development, there is a clear need to develop cryopreservation processes that operate at meaningful scale, and which integrate with other stages of an overall bioprocess. A well-integrated cryopreservation process enables the decoupling of commercially scaled cell therapy manufacture from final delivery and administration to patients. This in turn allows more cost-effective supply chain strategies for initial cell banking of isolated donor material, as well as the final product. The ideal result is an inventory of well-stored and

Abbreviations: CPA, cryoprotective agent; hMSC, human mesenchymal stem cell; ISCT, International Society for Cellular Therapy; pNNP, p-nitrophenyl phosphate; MoA, mechanism of action.

* Corresponding author.
E-mail address: k.coopman@lboro.ac.uk (K. Coopman).

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consistent cell therapy product, which can be globally distributed and administered at an affordable cost.

Human mesenchymal stem cells (hMSCs) are the most likely candidates for early successful human stem cell therapies, with hundreds of clinical trials evaluating their therapeutic potential [39]. MSCs are multipotent; relatively easy to isolate; have the capacity for self-renewal and proliferation and are undergoing assessment for a number of therapeutic indications including immunological, cardiac and musculoskeletal conditions [17,28]. From a manufacturing perspective, clear definition of critical quality attributes and a link to a therapeutic mechanism of action (MoA) is necessary. Unfortunately, the complexity of MSCs and diversity of patients and individual disease progression has resulted in a lack of clarity around MoA for these therapies [4]. A variety of potency assays have been developed for some therapeutic indications, but at present there is no consensus on which should be used for a given cell type and therapeutic indication. The International Society for Cellular Therapy (ISCT) recommends a minimal hMSC specification as being adherent to culture plastic; positive ($\geq$95%) for the expression of CD73, CD90 and CD105, negative ($\leq$2%) for the expression of CD14, CD19, CD34, CD45 and HLA-DR, and possessing the capacity to differentiate towards the chondrogenic, adipogenic and osteogenic lineages *in vitro* [8]. These guidelines continue to be widely used to specify identity assays for MSCs.

Using cryopreservation to decouple production and delivery of hMSC-based therapies is a popular strategy, as demonstrated by a 2013 survey highlighting that over 80% of MSC-based regulatory submissions used cryopreservation to store and transport the final product [23]. For example, Prochymal® (Mesoblast, USA), which has been conditionally approved for use in children with graft-versus-host disease in New Zealand and Canada, is stored in 15 mL Cryocyte bags with 10% (v/v) Me_2SO [14]. However, recent reports have suggested that cryopreserved cells do not perform as well therapeutically compared with freshly cultured cells [10,25]. Cryopreservation may be one of the reasons, as well as *in vitro* cell age, why several recent hMSC clinical trials have failed to reach their primary end-points, in contrast to earlier academic-led trials using freshly cultured cells. Given the technical nuances of cryopreservation, it is important to determine whether transient loss of cell function is an unavoidable feature of cryopreservation, or whether it is caused by aspects of the process that differ with process scale.

Me_2SO has been used as a cryoprotectant since the 1950s [21], but there is some evidence of rare but significant adverse effects in patients, including strokes, heart attacks and haemorrhages after infusion with bone marrow cryopreserved in 10% (v/v) Me_2SO [6]. Notably, not all adverse reactions can be directly attributed to Me_2SO [34] and the FDA Guidance for Human Somatic Cell Therapy and Gene Therapy [37] does not prohibit the use of Me_2SO as a CPA. However, there is a perception that minimizing exposure of cells to Me_2SO will mitigate infusion-related effects in patient and importantly, Me_2SO is also cytotoxic to human cells above 0 °C and has been linked to changes in differentiation capacity of stem cells [1,9,36,43,44]. The mechanisms of Me_2SO cytotoxicity are not well defined, but may be related to the enhanced diffusion of other molecules, including toxins, across cell membranes [42], or through destabilisation of normal ionic homeostasis. The use of Me_2SO and other permeating cryoprotectants must be managed carefully to minimise the transplantation of poorly functioning cells; dead cells; cell debris or potential cytotoxins into patients.

Cells are often processed in small batches for routine research using passive slow cooling devices (e.g. Mr. Frosty™ or CoolCell®), with modest cell densities around 1×10^6 cell/mL and cells being kept on ice until transfer into their cooling device. However, working cell banks for manufacturing can contains hundreds of vials and allogeneic therapies are expected to require lot sizes of 1×10^9 or 1×10^{10} cells to meet market demands at affordable cost, with each dose containing 5×10^7 - 2.5×10^8 cells [31]. Cryopreservation processes will therefore need to be in place to enable preservation of large numbers of small cryovials for banking and cryobags or larger vials (e.g. Crystal® or Daikyo Crystal Zenith® vials) for cell product packaging. In both instances, processing times will increase compared to lab scale and maintaining chilled temperatures during vial filling and the particulate testing required by USP <790> (Visible particulates in injections) for product vials is an operational burden. This means that the detrimental effects on cell quality of exposure to cryoprotectants like Me_2SO will likely impose limits to the achievable process scales for hMSC therapy manufacturing. For example, it has been reported that for recombinant baby hamster kidney (rBHK) cells, cryopreservation bags must be filled within a 2 h window to avoid adverse effects from Me_2SO exposure [15]. Similarly, Hunt et al. [16] showed that the "recovery index", a measure of viability from membrane integrity and colony-forming-ability after thaw, of CD34 positive cells can be reduced by as much as 50% when exposed to 25% (v/v) Me_2SO for up to an hour at 20 °C. Katkov et al. [18] showed that human embryonic stem cells lose around 50% expression of the vital Oct-4 marker after exposure to Me_2SO using standard protocols. These examples indicate that exposure of MSCs to Me_2SO will need to be limited in order to maintain therapeutically desirable cell characteristics. Furthermore, these tolerances will need to be defined and built into not just manufacturing but also clinical practice. For example, with product thawing and preparation for infusion or injection the implications of failures in a thawing protocol or device (e.g. long-term maintenance of the vial at > ambient temperature) and delays in preparing the patient for treatment must be considered in order to develop preparation guidelines for the product. To that end, we investigated the effect of Me_2SO on cell quality by exposing bone marrow derived hMSCs to this cryoprotectant for varying amounts of time i) without, ii) prior to, and iii) after employing standard freezing protocols. A maximum exposure time of 2 h at ambient temperatures prior to freezing or at 37 °C after freezing was chosen to represent worst case scenarios. Results are discussed in terms of the risk of product failure with cells consider to be 'overexposed' to Me_2SO once cell characteristics such as attachment capability and phenotype fall below pre-defined standards such as those set by the ISCT or FDA. Furthermore, the sensitivity of hMSCs to Me_2SO was compared to that of an osteosarcoma-derived cell line, HOS TE85, to establish whether hMSCs are more or less susceptible to Me_2SO-induced effects than similarly sized human cells.

2. Materials and methods

2.1. Cell culture

Osteosarcoma cells of the line HOS TE85 were acquired at passage 51 (ATCC, USA) and hMSCs derived from bone marrow at two passages post extraction were cultured as adherent monolayers in 25 cm^2 T-flasks (Nunc, UK). hMSCs had been extracted from ethically sourced fresh bone marrow aspirate obtained from Lonza (Lonza, Cologne AG) from a healthy donor after the patient provided informed consent. HOS TE85 cells were incubated in MEM medium (α-MEM, 1 g/l glucose with 10% (v/v) US origin FBS (Performance Plus) and 2 mM L-glutamine, (Thermo-Fisher, UK)), and hMSCs in DMEM medium (made up in the same way with 1 g/L glucose (Thermo-Fisher, UK) in place of α-MEM) in a humidified incubator at 37 °C, 5% CO_2. Passaging was done every 2–3 days (HOS) or 7 days (hMSC). A 100% medium exchange was done for hMSCs on day 4. Spent-medium was collected before passaging for

metabolite analysis. Cells were passaged with 0.25% (w/v) trypsin/EDTA. Samples were taken for cell counting and viability assessment, then the remaining cells were centrifuged at 220 g for 5 min. After aspirating the supernatant, the cell pellet was gently suspended in fresh medium. Cells were then used to seed new flasks at 2×10^4 cells/cm^2 (HOS) and 5×10^3 cells/cm^2 (hMSC).

2.2. Viable cell number

For HOS cells, 50 μL samples were taken and mixed with 50 μL of trypan blue stain (Sigma Aldrich, UK) then cells were counted manually using a haemocytometer and light microscope. A Nucleocounter NC-100 (Chemomotec, Denmark) was used to count hMSCs, which measures membrane integrity using propidium iodide (PI). These two counting methods have been previously found to be statistically similar [32].

2.3. Cryopreservation and thawing

For cryopreservation experiments, HOS cells were harvested after 2 days in culture and hMSC cells were harvested after 7 days in culture, using trypsin as described above in Section 2.1. After centrifugation, trypsin containing medium was aspirated and the resulting cell pellet was then suspended in freeze medium (10% (v/v) Me_2SO, 90% (v/v) FBS) at room temperature. 1 mL aliquots were loaded into 1.8 mL cryovials (Nunc, UK). Cryovials were placed into a CoolCell (BioCision, USA) and quickly transferred into a −80 °C freezer. This passively cooled the vials at approximately −1 °C/minute according to the manufacturer [2]. The total processing time from harvesting to loading of the CoolCell into the freezer was no more than one hour. After 4 h and no later than 24 h, cryovials were stored in the vapour phase of a liquid nitrogen cryostorage unit for at least 1 week.

Cryovials were retrieved from cryostorage then thawed in a 37 °C water bath for 4 min. Thawed cells were then diluted 1:1 in the vial, followed by 1:4 in 37 °C pre-warmed medium. Diluted cells were then centrifuged, counted and seeded into 25 cm^2 tissue culture flasks.

2.4. Experimental scheme

Cells were exposed to Me_2SO following three different schemes. For the 'just exposed' group, cells were suspended in a freeze medium at room temperature for 15, 30, 60 or 120 min, and then washed and cultured with fresh culture medium, followed by analysis for viability and attachment. In the 'exposure + freeze' group, cells were exposed to Me_2SO as per the "Just Exposed" group, transferred to cryovials and then frozen with a passive freezing device (1 °C/min) (CoolCell, BioCision), stored in the vapour phase of liquid nitrogen for one week and then thawed as normal. For the 'freeze + exposure' group, cells were frozen and stored as described in Section 2.3, but after thawing were left at 37 °C for 15, 30, 60 and 120 min before being washed in fresh medium, cultured and analysed. After one passage, all groups were analysed for growth, phenotype and metabolic activity, and then cultured for four further serial passages and their growth, phenotypic and metabolic activity was compared.

2.5. Alkaline phosphatase assay for HOS TE85 phenotype analysis

Cells at a density of 2.5×10^6/mL were lysed with two freeze/thaw cycles. In each cycle, cells were suspended in dH_2O, transferred to a −20 °C freezer for 24 h then warmed in a 37 °C water bath. 100 μL of cell lysate was transferred to a 48 well plate. Then, 300 μL of a working solution (1 (p-nitrophenyl phosphate substrate) pNPP tablet for every 5 mL, 1 part 20 mM $MgCl_2$, 1 part 1.5 M Alkaline buffer solution and 7 parts dH_2O) was added to the lysate. This solution was incubated for one hour in the dark at room temperature, before being quenched with 100 μL of 3M NaOH. 100 μL of the sample was transferred to a 96-well plate and measured with a plate reader at 405 nm absorbance. A standard curve of p-nitrophenol in the working solution without a pNPP tablet was used. All reagents were from Sigma-Aldrich, UK.

2.6. Five colour flow cytometry assay for hMSC phenotype analysis

This assay was carried out using a previously described protocol [5]. Harvested cells were suspended at 5×10^5 cells/mL in medium and 200 μL of cell suspension was added per well of a V bottomed 96-well plate (Corning, UK). The plate was centrifuged at 300 g for 5 min, the supernatant aspirated, and then the cells were re-suspended in 200 μL of flow cytometry staining buffer (R&D Systems, UK). The plate was centrifuged and the supernatant removed. As per Table 1, 5 μL of each antibody (BD Biosciences, UK) was then added to the cell pellets. The plate was then incubated in the dark at room temperature for 30 min, before being centrifuged. Stained cells were washed twice with staining buffer, then suspended in 200 μL of staining buffer. Data were obtained using a Guava® easyCyte 8HT flow cytometer (Merck Milipore, UK) equipped with excitation lasers at 488 nm and 640 nm and running guavaSoft™ InCyte™ (v2.5) acquisition software. For each sample, a minimum of 5000 (gated on forward/side scatter) events were recorded. Spectral overlap compensation was determined using fluorophore-conjugated anti-mouse Ig,κ antibodies. Analysis after acquisition and compensation was done with FlowJo v7.6.5. (Treestar, Inc, USA) software. To analyse for co-expression of all five markers, serial gating was used [5]. Gates were set firstly on the CD73^{+ve}/CD105^{+ve} population, subsequently on the HLA-DR^{-ve}/CD90^{+ve} and CD34^{-ve}/HLA-DR^{-ve} population (see Fig. 3E).

2.7. Metabolic analysis

1 mL of spent medium was taken at the end of each passage and before medium exchange for hMSCs. Glucose and lactate concentrations in the spent medium and fresh medium were measured using a Nova Bioprofile FLEX® (Nova Biomedical, UK). Net metabolite uptake and production by the cells from culture were inferred. The apparent yield of lactate from glucose [29] was calculated as follows:

$$Y\prime_{Lac/Glc} = \frac{\Delta[Lac]}{\Delta[Glc]}$$

where: $Y'_{Lac/Glc}$ = apparent lactate yield from glucose, Δ[Lac] = net lactate production over a specific time period, Δ[Glc] = net glucose consumption over the same time period.

Table 1
Antibodies used to detect surface markers. markers, their fluorescein and associated excitation and emission wavelength for the determination of hMSC phenotype.

Fluorophore	Target surface marker antibody	Max emission wavelength
PE-Cy5	CD34	667 nm
PE-Cy7	CD73	785 nm
APC	CD90	578 nm
PE	CD105	578 nm
FITC	HLA-DR	519 nm

2.8. One hour attachment efficiency

After exposure/thawing an additional flask was seeded to evaluate attachment efficiency. After one hours' incubation, cells were enzymatically detached from the culture vessel and counted. Cell counts were normalised to the known seeding density to determine attachment efficiency.

2.9. Statistics

All statistical data analysis was performed using the SPSS version 21 (IBM). Significance was determined using a One Way ANOVA test, followed by Dunnett's test for comparison with controls. Values of $p < 0.05$ were considered to be statistically significant. All data quoted represent the mean of two duplicates from three independent experiments, ± standard deviation (SD), unless otherwise stated.

3. Results and discussion

3.1. Impact of Me_2SO exposure on cell viability and cell attachment ability

HOS TE85s and early passage hMSCs were exposed to Me_2SO as described in Section 2.4 and membrane integrity as a proxy for viability was subsequently measured either immediately after exposure (just exposed group and freeze + exposure groups) or post-thaw (exposure + freeze). The viability of the HOS TE85 and hMSC cell populations decreased in a time-dependent manner under all three conditions of Me_2SO exposure (Fig. 1A and C). Viability fell to ~70% when cells were exposed to Me_2SO for 120 min once thawed after freezing (i.e. the 'freeze + exposure' group). It is noteworthy that the viability range exceeded the US FDA recommended lower limit for membrane for therapeutic applications [23,38]. These data indicate the importance of timing when carrying out the viability testing as part of release criteria as close to point of administration as possible, rather than at the immediate point of thaw (if these are to be different).

These results are similar to those reported by Hunt *et al* [16] who found that $CD34^+$ cells exposed to 25% (v/v) Me_2SO for up to 20 min at 2 °C showed negligible drop in viability, with loss of viability observed only with increasing time and temperature of exposure. However, the study is limited to Me_2SO exposure only, rather than the combination of exposure and freezing as shown in this work. Here we show that none of the just exposure groups (without cryopreservation) were statistically different from the non-exposed controls. This suggests that it is the combination of Me_2SO exposure and the cumulative complications of freezing and thawing, such as changes in osmolality during slow cooling and rewarming, which results in the loss of membrane integrity, rather than exposure to CPA per se.

It has been established that, although cells may appear viable upon thaw based on simple measures of viability like trypan blue exclusion, they may undergo delayed onset apoptosis [40]. For example, Aye et al. [1], showed that after 3 h CHO cells exposed to 20% Me_2SO still exhibit viability of over 90%, but after 24 h this was reduced to just 10%. Therefore, cell attachment one hour after seeding was also investigated as a potential indicator of cell survival. hMSC homing and engraftment *in vivo* might be necessary for sustained clinical function [19]. Normal attachment efficiency one hour after seeding is >85%, but both freezing and Me_2SO exposure time had impact on this response. The Frozen + Exposure group had the lowest attachment efficiency after two hours (Fig. 1C and D), with just 65% attachment for HOS TE85 cells, and 53% for hMSCs, a significant reductions of 21% and 46% respectively. Significant drops in attachment from the un-exposed controls were observed in the HOS TE85 120 min Freeze + Exposure group, and in hMSC 60 and 120 min Exposure + Freeze and Freeze + Exposure group. Cell

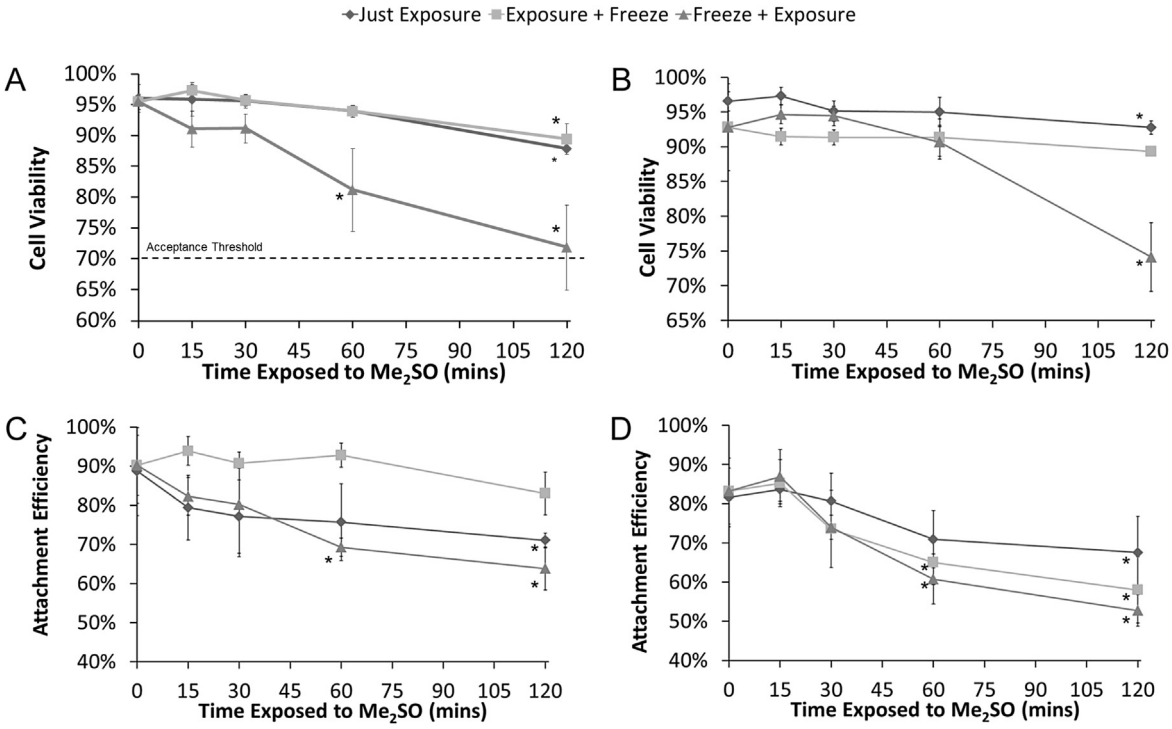

Fig. 1. Viability with respect to membrane integrity and attachment efficiency of (A,C) HOS TE85 and (B,D) hMSC cells after exposure to Me_2SO before and after cryopreservation for up to 120 min. Data are the mean ± SD derived from two duplicates from three independent experiments.

attachment has not been used extensively to assess cell quality, but it is one of the three key criteria for MSC identity proposed by Dominici et al., in 2006 [8], and perhaps the simplest to observe and quantify. Pal et al. [27] have previously noted unquantified abnormal cell attachment of stem cells after Me_2SO exposure for 24–48 h at low concentrations, 0.01% (v/v), 0.1% (v/v) and 1% (v/v) [27]. The data here also show that cell populations have an observed viability based on membrane integrity after thawing, but that this does not correlate to the number of cells that had adhered to tissue culture plastic one hour after seeding. The latter should be a more stringent metric of viability, as cell adhesion and spreading are reliant on active processes including actin filament formation [26] and energy metabolism. Furthermore, the proteolysis of cytoskeletal components, which maintain cell shape and are crucial for cell-surface attachment, is one of the first stages of delayed-onset apoptotic cell death commonly seen after cryopreservation [35]. Using attachment efficiency might therefore be a better candidate benchmark for release from the manufacturing site, as it relates better to MSC health and identity after recovery from cryopreservation. It was clear that using the same 70% threshold shows that cells reach this level of acceptability after around 1 h of exposure to Me_2SO and indicates that any longer than this would constitute overexposure as cell function is affected.

3.2. Impact of Me_2SO exposure on cell growth

Me_2SO has previously been shown to affect cell proliferation both negatively [35] and positively [30]. Therefore, to assess the potential for longer-term impact of Me_2SO exposure on cell quality, hMSC and HOS TE85 cell proliferation and basic metabolic profiles (Section 3.3) were assessed over multiple passages post-exposure. When growth over one passage post-thaw was investigated, there was no statistically significant effect of any Me_2SO treatments on HOS TE85 cells (Fig. 2A and B). The lack of a significant impact on growth rate indicates that in addition to the cells' quick recovery on thaw, Me_2SO has a limited effect on growth capability of osteosarcoma cells once washed away.

The hMSCs also showed a downward trend in growth with increasing exposure time (Fig. 2C), across all three treatment groups. However, only the Freeze + Exposure 60 and 120 min groups were statistically different from the non-exposed control. This lag in growth rate over the first passage post-thaw may be an important consideration for the therapeutic use of cryopreserved hMSCs, as engraftment and expansion in-vivo have direct bearing on the potential mechanisms of action clinically. Delayed outgrowth may also be an issue when hMSCs are banked in readiness to seed subsequent culture vessels for expansion as part of a cell therapy manufacturing process. The increased time required to achieve a desired cell yield directly increases costs. Furthermore, the loss of cells early in the process lowers the potential expansion headroom per banked vial, restricting the scale of manufacturing.

It is also important to consider growth over several passages in order to assess whether Me_2SO exposure has any sustained long-term impact on cells. This is important for cell therapies if the cells need to persist in the body for several days, or integrate into the host tissue and differentiate for their therapeutic effect. The HOS TE85 cells were cultured for five serial passages to see what effect Me_2SO had on long-term growth of these cells. Fig. 2C shows that over the 5 passages (at least 12 doublings), there was no significant difference between any of the treatment groups. Baseline experiments on HOS TE85 cells (data not shown) demonstrated that the exposed cells behaved in a similar fashion to the non-exposed groups. Consequently, we saw no long-term impact on HOS TE85 proliferative capacity or culture growth rate.

Human MSCs from the Just Exposure group were also cultured over 5 passages and as shown in Fig. 2D significant differences from the non-exposed control were observed in the 120 min group, further confirming that exposure of this time frame to Me_2SO causes a significantly detrimental effect on hMSC growth. Notably, a correlation of attachment to long term growth of 0.9704 was observed (Fig. 2E), indicating that a 1 h attachment efficiency assay reflected the longer-term growth potential for hMSCs which obviates the need for protracted outgrowth studies in future. Indeed, a short-term attachment efficiency assay has previously been used to successfully predict proliferative potential with human embryonic stem cells (hESCs) [24].

We therefore propose that cell attachment after 1 h is strongly correlated (Fig. 2E) with long-term expansion potential of the cell types cultured herein. This suggests that the impact of any delayed onset cell death is minimal if one wishes to culture cells *in vitro*. Clearly, there is potential for donor and processing variability, and this correlation should be explored during early product development to confirm its predictive value where cell expansion is a key part of a cell therapy bioprocess. However, it is not clear whether a correlation between in-vitro adhesion efficiency and expansion can be predictive of cell adhesion and retention in-vivo during cell therapy delivery. Mechanistically, this correlation is explained by the simple fact that these cells require adhesion to a surface before they can replicate. Fewer adherent cells mean a smaller effective seed population, with subsequent impact on long-term expansion potential in the absence of changes to the mean cell proliferation rate. This allows for a much quicker analysis process-weeks compared to 1 h for determining the long-term effect growth on cells (via colony forming unit counts).

3.3. Impact of Me_2SO exposure on cell metabolism

During addition and removal of cryoprotectant solutions, slow cooling and rewarming, cells are subjected to changes in osmolality and volume, with the latter being linked to changes in metabolism [20]. Furthermore, mitochondrial and metabolic changes have been shown to occur during stem cell differentiation [41], highlighting that process conditions that alter MSC metabolism could influence their function. As such, offline metabolic sampling during post-thaw expansion could provide early warning of detrimental changes. These could then be evaluated more closely with more laborious assays focusing on specific aspects of cell state. Accordingly, spent medium was analysed for glucose and lactate at each passage, and in the case of hMSCs at each medium exchange as well, to assess whether cells exposed to Me_2SO exhibited a change in their metabolic profile. From these data, the metabolic yield of lactate from glucose can be calculated and monitored. The maximum theoretical yield of lactate from glucose is 2 mol/mol. Values lower than this indicate glucose is being diverted elsewhere whereas values > 2 mol/mol indicate lactate is being generated from a non-glucose source [11].

For the HOS TE85 cells, a control value of glucose yield from lactate was shown to be 1.403 mol/mol (Fig. 3A) based on cells grown in continuous culture. None of the exposed groups were significantly different from the non-exposed control, indicating that there was no substantial change in glucose or lactate cell metabolism after freezing or Me_2SO exposure. However, there was variation across the hMSC groups (Fig. 3B). The hMSCs exposed after thaw for 60 min (Freeze + Exposure), and all three 120 min exposure groups were statistically significantly different from the non-exposed control. This indicates that 1 h or more of exposure to Me_2SO can influence the subsequent hMSCs metabolic profile during in-vitro expansion.

All exposed groups except the 15 min “Just Exposure” groups are above the 2 mol/mol theoretical yield limit, indicating the cells are

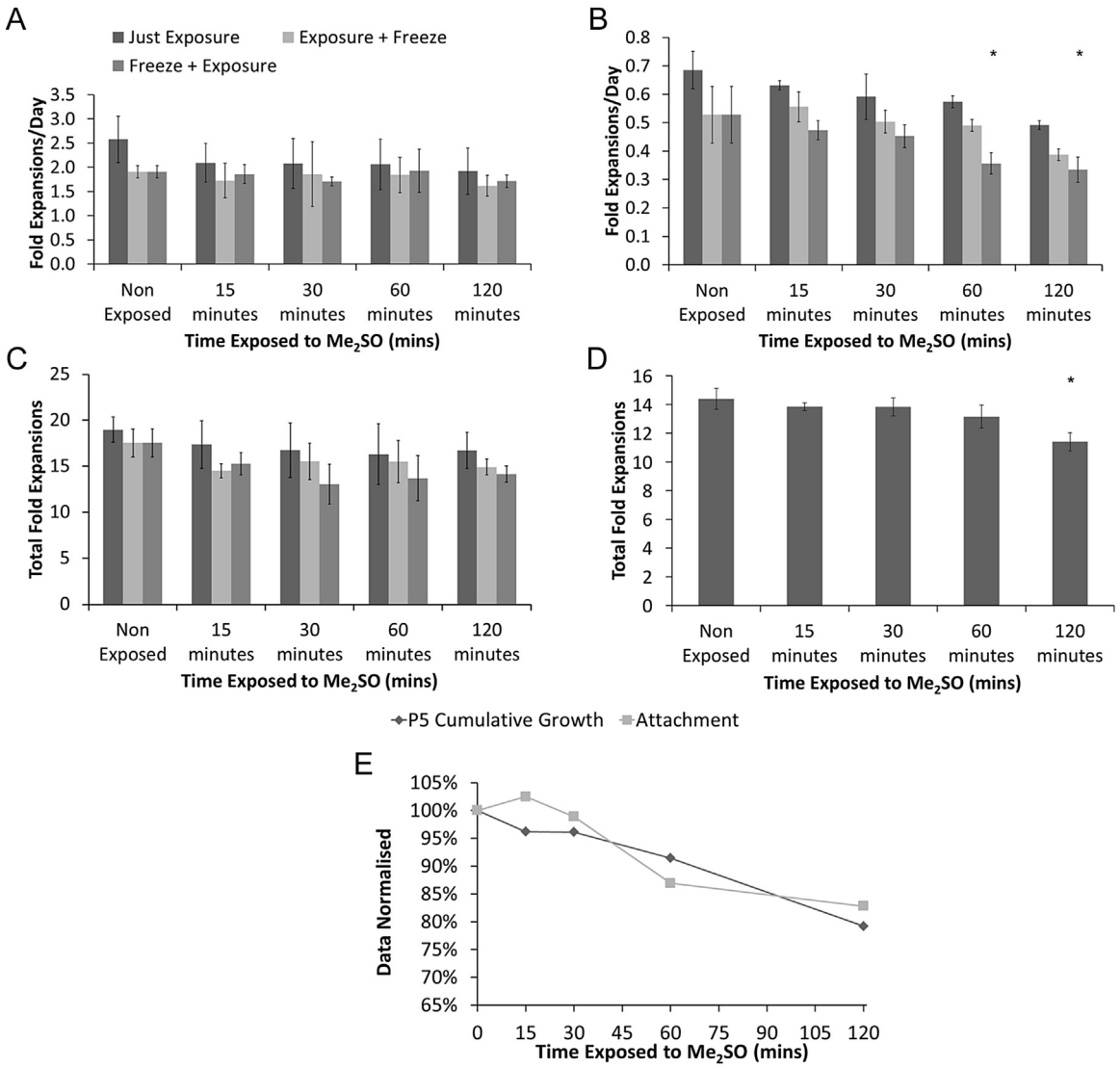

Fig. 2. Daily fold expansion rate over one passage and total fold expansion rate over 5 passages (A,C) HOS TE85 and (B,D) hMSC cells after exposure to Me_2SO before and after cryopreservation for up to 120 min. Data derived from two duplicates from three independent experiments. Error bars represent standard deviation.* indicates statistical significance ($p < 0.05$) from the respective non-exposed group. Error bars are standard deviation. E: Normalised data of 1 h attachment efficiency and five serial passages of hMSC cells after exposure to Me_2SO for up to 120 min and then washing.

converting an additional energy source to lactate, which is likely to be glutamine in the growth medium. This has been previously noted and proposed by Rafiq et al. [29] and perhaps indicates a less severe metabolic change than that experienced in the 60 min and 120 min groups. Furthermore, any change in metabolic profile could indicate that the cells are stressed or differentiating, and therefore cannot grow as efficiently, hence phenotype was assessed.

3.4. Impact of Me_2SO exposure on cell phenotype

Both cell types were cultured for one passage before phenotype analysis occurred, allowing any long-term phenotypic effect to be revealed. HOS TE85 phenotype was analysed by using an alkaline phosphatase (ALP) assay. ALP activity is indicative of a cells ability to mineralise bone, as it increases the local concentration of inorganic phosphate and decreases the concentration of extracellular pyrophosphate, which is an inhibitor of mineralisation [12]. While HOS TE85s are osteocarcoma cells and not true osteoblasts, baseline experiments (data not shown) showed that these cells retained some ALP activity in culture. Longer exposure to Me_2SO lowered ALP activity per cell, indicating a detrimental change in the osteoblastic phenotype (Fig. 3C). A reduced expression of ALP has previously been reported in pancreatic cells after exposure to Me_2SO [22].

When we consider the hMSCs, the expression of five extracellular markers was investigated; the co-expression of these is shown in Fig. 3D. There was a change in the phenotype away from the ISCT criteria [8] across all three groups as Me_2SO exposure time increases, with significant loss of phenotypic marker expression in the three most extreme conditions. Therefore, exposure to Me_2SO beyond an hour has a substantial negative effect on the hMSC phenotype after the cryopreservation process and thus constitutes overexposure. This may also indicate that Me_2SO is promoting differentiation of hMSCs. As Me_2SO has been used in conjunction with retinoic acid and 5-azacytidine to promote cardiac differentiation of human foetal liver-derived MSCs and can induce differentiation of human embryonic stem cells [7,18,27]. Although not

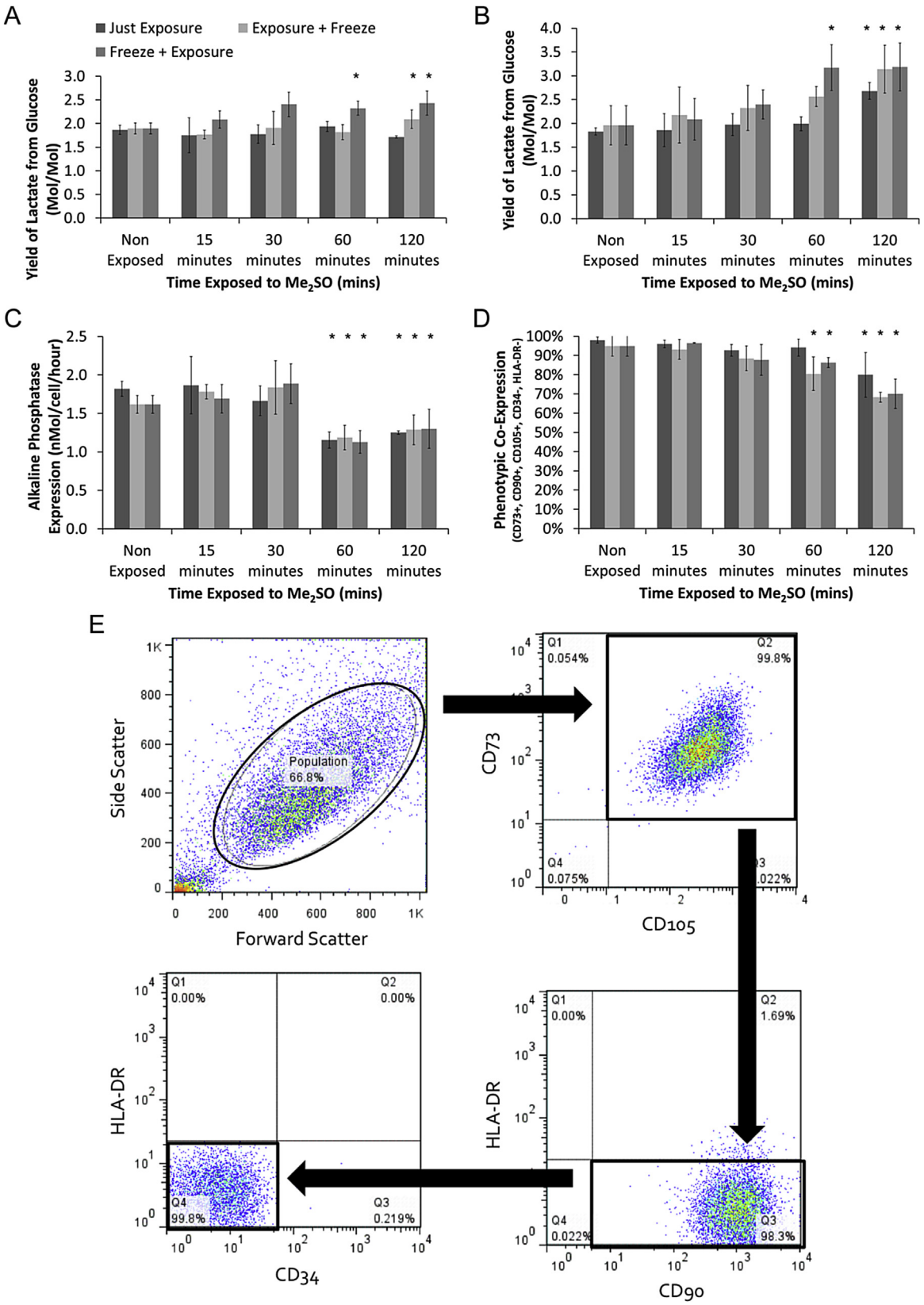

Fig. 3. Metabolic yield of lactate from glucose and phenotype data of (A,C) HOS TE85 and (B,D) hMSC cells after one passage in culture post exposure to Me_2SO before and after cryopreservation for up to 120min. Data derived from duplicates from three independent experiments. Error bars represent standard deviation.* indicates statistical significance ($p < 0.05$) from the respective non exposed groups. Phenotype Data derived from C: Alkaline Phosphatase Expression and D: Immunophenotype expression of CD73, CD105, CD34 and HLA-DR. E: Dot plots showing the serial gating method applied to Exposure + Freeze 15 min group.

statistically significant, the data also shows that the effect on phenotype was enhanced when cells were frozen. This indicates that factors such as osmotic stress and the changes in metabolism noted earlier in these groups may also affect phenotype. This is consistent with the fact that metabolic changes have previously been shown to occur during stem cell differentiation [41]. However, that phenotypic expression significantly differed from the hMSC standard after 120 min Me_2SO exposure, but cell viability on thaw was above the FDA's recommended minimum 70% viability criteria [4] raises the question whether on thaw viability is a reasonable release criteria in the absence of other indicators.

4. Conclusion

The successful cryopreservation of human cells is necessary to enable the production of off-the-shelf cell therapies, but challenges remain to retain cell functionality at commercial scale. Preventing changes to phenotype and metabolism is vital for cell therapeutics including hMSCs, which have been described by Caplan and Correa [3] as a "drugstore" for injury with the ability to establish a regenerative microenvironment through secreted protein factors, which regulate the local immune response at the injury site. If the cells are not what the physician or patient expects and cells have lost their therapeutic function or are unable to engraft, then patients will have been given at best an expensive placebo with added risk of harm.

However, working cell banks for manufacturing can contains hundreds of vials and with cellular therapeutics requiring $>10^9$ cells per dose [31] and with these increased volumes the cell-cryoprotectant exposure time will increase as the process scales from a laboratory to manufacturing environment. Limits on cryoprotectant exposure time, which will vary depending on the cell source as well as the upstream processing, may drastically reduce the scale of manufacture per batch. Hence we looked at two worst case scenarios using hMSCs and have shown that overexposure to Me_2SO, found here to occur with >1 h exposure prior to or after freezing, had a significant effect on cell metabolism and altered phenotype. We believe that these limits need to be accounted for when designing a larger scale manufacturing process and when training clinical staff in the thawing and use of cells preserved in Me_2SO to minimise the risk of product failure. For example, according to USP <790> (Visible Particulates in Injections) all vials of injectables need to be visually inspected for particulates and this a time consuming element of production, with chilling of vials prior to inspection adding to the burden due to condensation on the outside of the vials. Our data indicates that for products packaged in 1.5–5 mL cryovials, if a semi-automated vial filling systems such as the CellSeal® system from Cook RegenTec was being used to fill vials at a speed of 10 vials per 1–2 min, these would potentially not need to be chilled prior to particulate testing, especially if filling and testing could be co-located to ensure the vials were processed within this hour timeframe. Notably, similar trends were also seen with an osteoblast (HOS TE85) cell line indicating these results are not unique to MSCs. Defining the cell sensitivity to cryoprotectant exposure will be crucial to allow sensible decision-making with regard to larger-scale process design, including batch sizing and selection of equipment to meet processing requirements within a well-defined processing window. As there must be some exposure of cells to cryoprotectant post-thaw, controlling pre-freeze exposure is also essential to provide a safety-margin for exposure time during processing and removal of cryoprotectant after thawing, as the effects of cryoprotectant exposure will be cumulative. As such, tackling the issue of Me_2SO toxicity, by providing alternatives such as non-permeable sugars like Trehalose, in combination with permeabilising polymers [33], or negating the issue will be important for the commercialization of cell therapies.

Conflict of interest

None.

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